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(71) Applicant (for all designated States except US): UNIVERSITY OF SOUTHERN CALIFORNIA [US/US]; 3716 South Hope Street, Suite 313, Los Angeles, CA 90007-4344 (US).

(72) Inventor; and

(75) Inventor/Applicant (for US only): HEDMAN, Thomas, P. [US/US]; 25665 Frost Lane, Stevenson Ranch, CA 91381 (US).

(74) Agents: MCCLURE, Lawrence, J. et al.; Hogan & Hartson L.L.P., Biltmore Tower, 500 South Grand Avenue, Suite 1900, Los Angeles, CA 90071 (US).

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(54) Title: USE OF NON-TOXIC CROSSLINKING REAGENTS TO IMPROVE FATIGUE RESISTANCE AND REDUCE MECHANICAL DEGRADATION OF INTERVERTEBRAL DISC AND OTHER COLLAGENOUS TISSUES

(57) Abstract: A method of improving the resistance of collagenous tissue to mechanical degradation in accordance with the present invention comprises the step of contacting at least a portion of a collagenous tissue with an effective amount of a crosslinking reagent. The crosslinking reagent includes a crosslinking agent such as genipin and/or proanthocyanidin. Further, the crosslinking reagent may include a crosslinking agent in a carrier medium. The collagenous tissue to be contacted with the crosslinking reagent is preferably a portion of an intervertebral disc or articular cartilage. The contact between the tissue and the crosslinking reagent is effected by injections directly into the select tissue using a needle. Alternatively, contact between the tissue and the crosslinking reagent is effected by placement of a time-release delivery system such as a gel or ointment, or a treated membrane or patch directly into or onto the target tissue. Contact may also be effected by, for instance, soaking.

**USE OF NON-TOXIC CROSSLINKING REAGENTS TO IMPROVE FATIGUE  
RESISTANCE AND REDUCE MECHANICAL DEGRADATION OF  
INTERVERTEBRAL DISC AND OTHER COLLAGENOUS TISSUES**

5 Cross-Reference to Related Application

This application claims the benefit of U.S. Provisional Application No.  
60/316,287, filed August 31, 2001.

Background of the Invention

10 Field of the Invention

The present invention relates to a method for treatment of tissue, for  
example, collagenous tissue, where a deleterious mechanical loading environment  
contributes to the degradation of the tissue. More specifically, the present invention  
relates to a method for treatment of degenerated intervertebral discs to improve  
15 fatigue resistance, and to non-toxic crosslinking reagents that are effective fatigue  
inhibitors.

Description of the Related Art

Back pain and disability associated with spinal degeneration and  
instability continue to be one of the costliest and most prevalent health problems in  
20 western civilization. Current treatments for spinal instability and low-back pain,  
including spinal fusion, are generally ineffective in slowing the progression of  
degeneration. Epidemiological and morphological studies have shown that the  
capacity of spinal tissue to withstand repetitive loading is one critically important  
factor in the progression of spinal osteoarthritis (Magora 1972, Kelsey 1975,  
25 Frymoyer 1983, Videman 1990).

The organization of collagen and proteoglycans within the  
intervertebral disc plays an important role in determining the biomechanical  
properties of the disc. Biochemical alterations in the structure of the annular

matrix affect the disc's durability, that is, its ability to withstand repetitive mechanical loading. Previous studies have shown that nonreducible pyridinoline cross links are predominant in adult cartilage, bone, and intervertebral discs and these collagen crosslinks are thought to be critical for the structural integrity  
5 (enzymatic and mechanical) of adult connective tissue (Burgeson and Nimni, 1992, Eyre, 1988). Pentosidine crosslinking has been shown to increase with age in articular cartilage and intervertebral discs (Bank 1998, Pokharna 1998).

A role for naturally occurring crosslinks in stabilizing degenerating discs has been suggested. Duance (1998) noted that while the nonenzymic derived  
10 crosslink pentosidine showed an expected age related increase, its level was lower in the more severely degenerated samples. It may be that age related tissue changes -- i.e. micro-damage accumulation -- combined with inadequate levels of crosslinks made these discs more vulnerable to mechanical degradation. Age related crosslinks (pentosidine) have been shown to increase the strength and  
15 stiffness of articular cartilage (Chen 2001) while age related microdamage accumulation would act to decrease strength and stiffness. With regard to viscoelastic properties, Lee (1989) found that aldehyde fixation (crosslinking) reduced stress-relaxation and creep in bovine pericardium, while fatigue loading produced an increase in stress-relaxation and creep in our preliminary testing of  
20 intervertebral discs.

Crosslinking reagents are capable of improving the tensile properties of collagen-based biomaterials. Osborne et al (1998) found mechanical strength of acellular collagen gels was most improved using a combination of crosslinking agents. Other researchers have also found that crosslinking treatments can  
25 increase the strength of collagenous tissues (Wang 1994, Chachra 1996, Sung 1999, Zeeman 1999). Sung (1999) found that a naturally occurring cross linking agent, genipin, provided greater ultimate tensile strength and toughness when compared with other crosslinking reagents. Genipin also demonstrated significantly less cytotoxicity compared to other more commonly used crosslinking agents. However

it also stood out in a negative sense with regard to eliminating tissue anisotropy in bovine pericardium. Several researchers have stated their expectation that crosslinking of collagenous tissue may make the tissue more prone to fatigue failure (Bank 1998, Chen 2001, Kerin 2001). However, it is believed that the opposing  
5 view—that crosslinking collagenous tissue may actually benefit fatigue resistance—has not been recorded in the medical literature. It is believed that collagen crosslinks may act as sacrificial bonds to protect collagenous tissues by dissipating energy and improving fatigue resistance.

Fatigue is a weakening of a material due to repetitive applied stress.  
10 Fatigue failure is simply a failure where repetitive stresses have *weakened* a material such that it fails below the original ultimate stress level. In bone, two processes -- biological repair and fatigue -- are in opposition, and repair generally dominates. In the intervertebral disc, the prevalence of mechanical degradation of the posterior annulus (Osti 1992) suggests that fatigue is the dominant process.  
15 Active tissue response (adaptation, repair) does not play a strong role in the case of mature intervertebral disc annular material. As a principally avascular structure, the disc relies on diffusion for nutrition of its limited number of viable cells. Age related changes interfere with diffusion presumably contributing to declining cell viability and biosynthetic function (Buckwalter et al. 1993, Buckwalter 1995). Age  
20 related decline in numbers of cells and cell functionality compromises the ability of the cells to repair mechanical damage to the matrix. While regeneration of the matrix in the nucleus following enzymatic degradation has been accomplished, albeit inconsistently (Deutman 1992), regeneration of functional annular material has not yet been realized.

25 Combined with this limited potential for repair or regeneration, studies have shown that posterior intervertebral disc tissue is vulnerable to degradation and fatigue failure when subjected to non-traumatic, physiologic cyclic loads. Prior work has shown deterioration in elastic-plastic (Hedman 99) and viscoelastic (Hedman 00) material properties in posterior intervertebral disc tissue

subjected to moderate physiological cyclic loading. Cyclic load magnitudes of 30% of ultimate tensile strength produced significant deterioration of material properties with as little as 2000 cycles. Green (1993) investigated the ultimate tensile strength and fatigue life of matched pairs of outer annulus specimens. They found that fatigue failure could occur in less than 10,000 cycles when the vertical tensile cyclic peak exceeded 45% of the ultimate tensile stress of the matched pair control. In addition, Panjabi et al (1996) found that single cycle sub-failure strains to anterior cruciate ligaments of the knee alter the elastic characteristics (load-deformation) of the ligament. Osti (1992) found that annular tears and fissures were predominantly found in the posterolateral regions of the discs. Adams (1982) demonstrated the propensity of slightly degenerated discs to prolapse posteriorly when hyperflexed and showed that fatigue failure might occur in lumbar discs as the outer posterior annulus is overstretched in the vertical direction while severely loaded in flexion. In an analytical study, interlaminar shear stresses, which can produce delaminations, have been found to be highest in the posterolateral regions of the disc (Goel 1995). These prior data indicate: 1) the posterior disc and posterior longitudinal ligament are at risk of degenerative changes, and that 2) the mechanism of degeneration can involve flexion fatigue.

To date, however, no treatments capable of reducing mechanical degradation to collagenous tissues currently exist. In fact, no other collagenous tissue fatigue inhibitors have been proposed. A need therefore exists for a method for improving the resistance of collagenous tissues in the human body to fatigue and for reducing the mechanical degradation of human collagenous tissues, in particular, the posterior annulus region of the intervertebral disc.

25

#### Summary of the Invention

It is one object of the present invention to provide a method of improving the resistance of collagenous tissues in the human body to mechanical degradation by contacting the tissue with crosslinking reagents.

It is another object of the present invention to provide a method of curtailing the progressive mechanical degradation of intervertebral disc tissue by enhancing the body's own efforts to stabilize aging discs by increasing collagen crosslinks.

5           It is another object of the present invention to provide a method that uses crosslinking reagents with substantially less cytotoxicity compared to common aldehyde fixation agents in order to facilitate direct contact of these reagents to tissues in the living human body.

10           It is another object of the present invention to increase the crosslinking of disc annular tissue by directly contacting living human disc tissue with appropriate concentrations of a non-toxic crosslinking reagent (or a mixture of crosslinking reagents) such as genipin (a geniposide) or proanthrocyanidin (a bioflavonoid).

15           It is another object of the present invention to provide a treatment method for minimally invasive delivery of the non-cytotoxic crosslinking reagent such as injections directly into the select tissue using a needle or placement of a time-release delivery system such as a carrier gel or ointment, or a treated membrane or patch directly into or onto the target tissue.

20           It is another object of the present invention to a composition composed of non-toxic crosslinking reagents that can be used as effective fatigue inhibitors.

25           In accordance with the present invention, there is provided a method for treatment of tissues where a deleterious mechanical loading environment contributes to the degradation of the tissue. The deleterious mechanical loading environment may consist of normal physiological repetitive loading, otherwise known as fatigue. The present invention provides a method for treatment of degenerated intervertebral discs to improve fatigue resistance. The present invention also provides non-toxic crosslinking compositions that are effective fatigue inhibitors.

A method of improving the resistance of collagenous tissue to mechanical degradation in accordance with the present invention comprises the step of contacting at least a portion of a collagenous tissue with an effective amount of a crosslinking reagent. The crosslinking reagent includes a crosslinking agent such as genipin and/or proanthrocyanidin. Further, the crosslinking reagent may include a crosslinking agent in a carrier medium. The collagenous tissue to be contacted with the crosslinking reagent is preferably a portion of an intervertebral disc or articular cartilage. The contact between the tissue and the crosslinking reagent is effected by injections directly into the select tissue using a needle. Alternatively, contact between the tissue and the crosslinking reagent is effected by placement of a time-release delivery system such as a gel or ointment, or a treated membrane or patch directly into or onto the target tissue. Contact may also be effected by, for instance, soaking or spraying.

#### 15 Description of the Figures

FIGURE 1 is a graph of relaxation (N) v. numbers of cycles showing the effect of genipin crosslinking treatments ( $G1=0.033$  g/mol,  $G2=0.33$  g/mol) on posterior intervertebral disc stress relaxation.

FIGURE 2 is a graph of Brinnell's hardness index v. numbers of cycles showing the effect of genipin crosslinking treatments ( $G1=0.033$  g/mol,  $G2=0.33$  g/mol) on posterior intervertebral disc hardness or resistance to penetration.

#### Detailed Description of the Invention

The present invention provides a method of improving the resistance of collagenous tissues in the human body to mechanical degradation comprising the step of contacting at least a portion of a collagenous tissue with an effective amount of a crosslinking reagent. The method of the present invention also provides a method of curtailing the progressive mechanical degradation of intervertebral disc

tissue by enhancing the body's own efforts to stabilize aging discs by increasing collagen crosslinks. This mechanical degradation may be in response to physiologic levels of repetitive loading.

The crosslinking reagent of the present invention is not particularly limited.

5 Any crosslinking reagent known to be substantially non-cytotoxic and to be an effective cross-linker of collagenous material may be used. The crosslinking reagent is required to be substantially non-cytotoxic in order to facilitate direct contact of the crosslinking agent to tissues in the living human body. Preferably, the crosslinking reagent exhibits substantially less cytotoxicity compared to common  
10 aldehyde fixation agents. More preferably, a non-cytotoxic crosslinking reagent is used.

The crosslinking reagent includes at least one crosslinking agent. The crosslinking agent chosen in accordance with the present invention is an effective cross-linker of collagenous material. When used in a cross-linking reagent, an  
15 effective crosslinker is one that increases the number of crosslinks in the collagenous tissue when the crosslinker is brought into contact with a portion of the collagenous tissue. An effective crosslinker improves the fatigue resistance of the treated tissue, reduces material property degradation resulting from repetitive physiologic loading, or reduces the increase of viscoelastic properties of the treated  
20 tissue due to fatigue loading. Likewise, an effective crosslinker may reduce the decrease in elastic-plastic properties due to fatigue loading of the treated tissue. In one embodiment of the present invention, the crosslinking agent is Genipin, a non-toxic, naturally occurring crosslinking agent. Genipin is obtained from its parent compound, geniposide, which may be isolated from the fruits of *Gardenia*  
25 *jasminoides*. Genipin may be obtained commercially from Challenge Bioproducts Co., Ltd., 7 Alley 25, Lane 63, TzuChiang St. 404 Taichung Taiwan R.O.C., Tel 886-4-3600852. In another embodiment of the present invention, the crosslinking agent is a bioflavonoid, and more specifically, the bioflavonoid is proanthocyanidin. A mixture containing proanthocyanidin can be obtained as MegaNatural™ Gold



from Polyphenolics, Inc, 22004 Rd. 24, Medera, CA 93638, Tel 559-637-5961. More than one crosslinking agent may be used.

5 The crosslinking reagent may include a carrier medium in addition to the crosslinking agent. The crosslinking agent may be dissolved or suspended in the carrier medium to form the crosslinking reagent. In one embodiment, a crosslinking agent is dissolved in a non-cytotoxic and biocompatible carrier medium. The carrier medium is required to be substantially non-cytotoxic in order to mediate the contact of the crosslinking agent to tissues in the living human body without substantial damage to the tissue or surrounding tissue. Preferably, the carrier  
10 medium chosen is water, and more preferably, a saline solution. Preferably, the pH of the carrier medium is adjusted to be the same or similar to the tissue environment. Even more preferably, the carrier medium is buffered. In one embodiment of the present invention, the carrier medium is a phosphate buffered saline (PBS).

15 When the crosslinking agent is dissolved in a carrier medium, the concentration of the crosslinking agent in the carrier medium is not particularly limited. The concentration may be in any amount effective to increase the crosslinking of the tissue while at the same time remaining substantially noncytotoxic. When the crosslinking agent is genipin, the concentration of the  
20 crosslinking agent is preferably greater than 0.033% in PBS (wt %), and more preferably, about 0.33% in PBS (wt %).

In accordance with the present invention, the crosslinking reagent is brought into contact with a portion of a collagenous tissue. As used herein, collagenous tissue is defined to be a structural or load supporting tissue in the body comprised  
25 of a substantial amount of collagen. Examples would include intervertebral disc, articular cartilage, ligament, tendon, bone, and skin. In general, the portion of the collagenous tissue to be brought into contact with the crosslinking reagent is the portion of the tissue that is subject to loading. Further, where at least some degradation of the collagenous tissue has occurred, the portion of the tissue to be

contacted with the crosslinking reagent is at least the portion of the tissue that has been degraded. Preferably, the entire portion that is subject to loading or the entire portion that is degraded is contacted with the crosslinking reagent. Further, the tissue adjacent the portion of collagenous tissue subject to the loading may also be  
5 contacted with the crosslinking reagent.

The collagenous tissues that are particularly susceptible for use in accordance with the present invention include intervertebral discs and articular cartilage. Where the collagenous tissue is an intervertebral disc, the portion of the intervertebral disc that is preferably contacted by the crosslinking reagent is the  
10 posterior and posterolateral annulus fibrosis.

The selected portion of the collagenous tissue must be contacted with an effective amount of the non-toxic crosslinking reagent. An "effective amount" is an amount of crosslinking reagent sufficient to have a mechanical effect on the portion of the tissue treated. Specifically, an "effective amount" of the crosslinking reagent  
15 is an amount sufficient to improve the fatigue resistance of the treated tissue, reduce material property degradation resulting from repetitive physiologic loading, or reduce the increase of viscoelastic properties of the treated tissue due to fatigue loading, or reduce the decrease of elastic-plastic properties of the treated tissue due to fatigue loading. An effective amount may be determined in accordance with the  
20 viscoelastic testing and/or the elastic-plastic testing described herein with respect to Examples 1 and 2.

The method of the present invention includes contacting at least a portion of the collagenous tissue with an effective amount of the crosslinking reagent. The contact may be effected in a number of ways. Preferably, the contacting of  
25 collagenous tissue is effected by a means for minimally invasive delivery of the non-cytotoxic crosslinking reagent. Preferably, the contact between the tissue and the crosslinking reagent is effected by injections directly into the select tissue using a needle. Preferably, the contact between the tissue and the crosslinking reagent is effected by injections from a single or minimum number of injection locations.

Preferably, an amount of crosslinking solution is injected directly into the targeted tissue using a needle and a syringe. Preferably, a sufficient number of injections are made along the portion of the tissue to be treated so that complete coverage of the portion of the collagenous tissue to be treated is achieved.

- 5           Alternatively, contact between the tissue and the crosslinking reagent is effected by placement of a time-release delivery system directly into or onto the target tissue. One time-released delivery system that may be used is a treated membrane or patch. A reagent-containing patch may be rolled into a cylinder and inserted percutaneously through a cannula to the tissue sight, unrolled and using a  
10   biological adhesive or resorbable fixation device (sutures or tacks) be attached to the periphery of the targeted tissue.

          Another time-released delivery system that may be used is a gel or ointment. A gel or ointment is a degradable, viscous carrier that may be applied to the exterior of the targeted tissue.

- 15           Contact also may be effected by soaking or spraying, such as intra-capsular soaking or spraying, in which an amount of crosslinking solutions could be injected into a capsular or synovial pouch.

- It should be noted that the methods and compositions treated herein are not required to permanently improve the resistance of collagenous tissues in the human  
20   body to mechanical degradation. Assuming that a person experiences 2 to 20 upright, forward flexion bends per day, the increased resistance to fatigue associated with contact of the collagenous tissue with the crosslinking reagent, may, over the course of time, decrease. Preferably, however, the increased resistance to fatigue lasts for a period of several months to several years without physiologic  
25   mechanical degradation. Under such circumstance, the described treatment can be repeated at the time periods sufficient to maintain an increased resistance to fatigue resistance. Using the assumption identified above, the contacting may be repeated periodically to maintain the increased resistance to fatigue. For some treatment, the time between contacting is estimated to correspond to approximately

1 year for some individuals. Therefore, with either a single treatment or with repeated injections/treatments, the method of the present invention minimizes mechanical degradation of the collagenous tissue over an extended period of time.

## 5      Examples 1 and 2

Thirty-three lumbar intervertebral joints were obtained from ten four-month-old calf spines. The intervertebral joints were arbitrarily divided into 3 groups: untreated controls-12 specimens, Genipin treatment 1 (G1)-6 specimens, and Genipin treatment 2 (G2)-13 specimens. The G1 treatment involved 72 hours of soaking the whole specimen in PBS with a 0.033% concentration of Genipin. Similarly the G2 treatment involved 72 hours of soaking whole specimens in PBS with 0.33% concentration of Genipin. 0.33% Genipin in PBS is produced by dilution of 50 ml of 10x PBS (Phosphate Buffered Saline) with distilled water by a factor of 10 to give 500 ml (500 gm) of PBS and mixing in 1.65 grams of genipin to produce the 0.33 % (wt%, gm/gm) solution. Previous testing with pericardium and tendon tissue samples demonstrated the reduction of tissue swelling (osmotic influx of water into the tissue) resulting from crosslinking the tissue. Some controls were not subjected to soaking prior to fatigue testing. Others were soaked in a saline solution for 72 hours. Water mass loss experiments were conducted to establish the equivalency of outer annulus hydration between the genipin soaked and 0.9 % saline soaked controls. The selection of treatments was randomized by spine and level. The vertebral ends of the specimens were then potted in polyurethane to facilitate mechanical testing.

Indentation testing and compression/flexion fatigue cycling were carried out in the sequence presented in Table 1.

Table 1. Experimental protocol

Measurement Sequence	Measurement	Location
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1	Stress Relaxation	Center of the Posterior Annulus
2	Hardness	Center of the Posterior Annulus
3000 Compression/Flexion Fatigue Cycles		
3	Stress Relaxation	4 mm Lateral to Center
4	Hardness	Center of the Posterior Annulus
Additional 3000 Compression/Flexion Fatigue Cycles		
5	Stress Relaxation	4 mm Lateral to Center (Opposite Side)
6	Hardness	Center of the Posterior Annulus

At the prescribed points in the loading regimen, indentation testing was used to find viscoelastic properties as follows. Stress relaxation data was gathered by ramp loading the 3 mm diameter hemi-spherical indenter to 10 N and subsequently holding that displacement for 60 s, while recording the resulting decrease in stress, referred to as the stress relaxation. Indentation testing was also utilized to determine elastic-plastic properties by calculating a hardness index (resistance to indentation) from ramp loading data. Prior to recording hardness measurements, the tissue is repeatedly indented 10 times (60 s/cycle, to the displacement at an initial 10 N load).

This test protocol is based on two principles. First, viscoelastic effects asymptotically decrease with repeated loading. Secondly, hardness measurements are sensitive to the loading history of the tissue. However this effect becomes negligible following 10 loading cycles. In order to minimize these effects, viscoelastic data (stress relaxation) was collected from tissue that had not previously been indented. Alternately, elastic-plastic data (hardness) was collected from tissue that had been repeatedly loaded (preconditioned). In this case, repetitive indentation was intended to reduce the undesired effects of the changing viscoelastic properties, namely lack of repeatability, on hardness measurements. These testing procedures were derived from several preliminary experiments on the repeatability of the measurements with variations of loading history and location.

Following initial indentation testing, the specimen was loaded repetitively in flexion-compression at 200 N for 3000 cycles at a rate of 0.25 Hz. The load was applied perpendicularly to the transverse plane, 40 mm anterior to the mid-point of the specimen in the transverse plane. A second set of indentation testing data is then collected following fatigue cycling. This procedure was followed for two fatigue loading cycles. During all testing, the specimens were wrapped in saline wetted gauze to maintain their moisture content. Fatigue cycling and non-destructive indentation testing were carried out on an MTS 858.02 biaxial, table-top, 10kN capacity servo-hydraulic materials test station (MTS, Eden Prairie, MN), with the MTS Test Star data acquisition system. Several statistical measures were calculated to evaluate the significance of the results. A nested two-way analysis of variance (ANOVA) was utilized to confirm effects due to treatment and number of fatigue cycles. Due to the non-parametric nature of the data, the Mann-Whitney non-parametric rank-sum test was used to assess the null hypotheses that the treatment did not affect: 1) the pre-cycling mechanical parameters of the tissue, or 2) the amount of change (degradation) in elastic-plastic and viscoelastic mechanical parameters due to fatigue loading. The confidence level for statistical significance was set at  $p < 0.05$ .

Nested two-way ANOVA analysis determined that both viscoelastic (relaxation) and elastic-plastic (hardness) mechanical parameters were independently affected by fatigue cycling and by treatment type. These statistical results are presented in Table 2.

The relaxation test results are presented graphically in Figure 1. There was an initial shift downward of the relaxation curve caused by the crosslinking treatment. This would represent a beneficial effect as higher stress relaxation would be associated with more severely degraded tissue (Lee 1989). The initial pre-fatigue relaxation of the G1 and G2 treatment groups were 26% and 19% less than ( $p = 0.009$  and  $p = 0.026$ ) the pre-fatigue relaxation of the controls respectively. There was also dramatic improvement in fatigue resistance as

demonstrated by the change in relaxation after 6000 non-traumatic loading cycles. The change in relaxation due to 6000 fatigue cycles for the G2 treated discs was less than a third of the change in the controls ( $p=0.044$ ). However, the lesser concentration of Genepin did not bring about the same improvement in fatigue resistance.

The hardness test results are presented graphically in Figure 2. There is an initial shift upward of the hardness data caused by the G2 crosslinking treatment. This would represent a beneficial effect as loss of hardness would signal a loss of structural integrity in the tissue. The initial pre-fatigue hardness of the G2 treatment group was 17% greater than that of the control group ( $p=0.026$ ). However this beneficial effect appears to have eroded prior to 3000 fatigue cycles and the change in hardness between 3000 and 6000 cycles is essentially the same for the two groups (G2= -0.94, Control= -1.01).

Table 2. Results of nested two-way ANOVA analysis

Material Property	Factor	F-Value	Probability
Stress Relaxation	Treatment	16.060	1.085E-06
	Fatigue Cycling	9.676	2.500E-03
	Interaction	1.402	2.515E-01
Hardness	Treatment	20.023	6.405E-08
	Fatigue Cycling	5.898	1.710E-02
	Interaction	4.228	1.760E-02

The data presented above quantifies the elastic and viscoelastic mechanical degradation of intervertebral disc tissue due to repetitive, non-traumatic loading. The results of these experiments establish that non-toxic crosslinking reagents reduce the fatigue-related degradation of material properties in a collagenous tissue -- namely the intervertebral disc. More than a three-fold reduction in viscoelastic degradation was brought about by soaking the calf disc tissue in 0.33 g/mol concentration of genipin. The tested formulation was unable to

sustain an improvement in the elastic mechanical properties (hardness) to 3000 test cycles.

Accurately estimating the length of time it would take an average person to experience a comparable amount of wear and tear on their spinal discs is difficult. Certainly, in addition to the mechanical degradation imposed by the described testing, there is the added -- "natural" -- degradation of these dead tissues due to the testing environment. The non-loaded controls showed this "natural" degradation of material properties to be insignificant. Measures were taken to minimize this natural degradation by keeping the specimens moist throughout the testing and by accelerating the loading frequency. At the same time, loading frequency was kept within physiologic limits to prevent tissue overheating. It should be noted that these measures constitute standard protocol for in vitro mechanical testing of cadaveric tissues. Assuming that a person experiences 2 to 20 upright, forward flexion bends per day, these data roughly correspond to several months to several years of physiologic mechanical degradation.

The described treatment could be repeated at the time periods represented by, for instance, 3000 fatigue cycles at this load magnitude. Using the assumption identified above, this number of cycles may be estimated to correspond to approximately 1 year for some individuals. Therefore, with either a single treatment or with repeated injections/treatments, an individual may be able to minimize mechanical degradation of their intervertebral discs over an extended period of time. Another option would involve a time-release delivery system such as a directly applied treated patch, a gel or ointment.

The invention has been described in terms of certain preferred and alternate embodiments which are representative of only some of the various ways in which the basic concepts of the invention may be implemented. Certain modification or variations on the implementation of the inventive concepts which may occur to those of ordinary skill in the art are within the scope of the invention and equivalents, as defined by the accompanying claims.



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## I CLAIM:

1. A method of improving the resistance of collagenous tissue to mechanical degradation comprising the steps of:  
contacting at least a portion of a collagenous tissue with an effective amount  
5 of a crosslinking reagent.
2. The method of claim 1, wherein the crosslinking reagent includes genipin.
3. The method of claim 1, wherein the crosslinking reagent includes  
10 proanthrocyanidin.
4. The method of claim 1, wherein the crosslinking reagent includes a crosslinking agent in a carrier medium.
- 15 5. The method of claim 4, wherein the crosslinking agent is selected from the group consisting of genipin and proanthrocyanidin and the carrier medium is a buffered saline solution.
6. The method of claim 4, wherein the crosslinking agent is genipin, the  
20 carrier medium is a buffered saline solution, and a concentration of the genipin in the buffered saline solution is greater than 0.033%.
7. The method of claim 1, wherein the collagenous tissue is an  
intervertebral disc.  
25
8. The method of claim 1, wherein the collagenous tissue is articular cartilage.

9. The method of claim 1, wherein the contact between the collagenous  
30 tissue and the crosslinking reagent is effected by injections directly into the portion  
of the collagenous tissue with a needle.

10. The method of claim 1, wherein the contact between the collagenous  
tissue and the crosslinking reagent is effected by placement of a time-release  
35 delivery system directly into or onto the portion of the collagenous tissue.

11. The method of claim 10, wherein the time-release delivery system is a  
gel or ointment.

40 12. The method of claim 10, wherein the time-release delivery system is a  
treated membrane or patch.

13. The method of claim 10, wherein the time-release delivery system is a  
treated patch.  
45

14. The method of claim 1, wherein the contact between the collagenous  
tissue and the crosslinking reagent is effected by soaking.

15. The method of claim 1, further comprising the step of:  
50 periodically re-contacting the portion of the collagenous tissue with an  
effective amount of a crosslinking reagent.

16. A crosslinking reagent comprising a crosslinking agent dissolved in a  
carrier medium wherein the crosslinking agent is present in an amount effective to  
55 improve the fatigue resistance of a portion of a collagenous tissue and wherein the  
crosslinking agent and the carrier medium are substantially non-cytotoxic.

17. The crosslinking reagent according to claim 16, wherein the crosslinking agent is selected from the group consisting of genipin and  
60 proanthrocyanidin.

18. A method of improving the resistance of collagenous tissue to mechanical degradation comprising the steps of:  
preparing a crosslinking reagent by dissolving a crosslinking agent in a  
65 carrier medium; and  
contacting at least a portion of a collagenous tissue with an effective amount of the crosslinking reagent .

19. The method of claim 18, wherein the contact between the collagenous  
70 tissue and the crosslinking reagent is effected by injections directly into the portion of the collagenous tissue with a needle.

20. The method of claim 18, wherein the contact between the collagenous tissue and the crosslinking reagent is effected by placement of a time-release  
75 delivery system directly into or onto the portion of the collagenous tissue.

21. The method of claim 18, wherein the contact between the collagenous tissue and the crosslinking reagent is effected by soaking.

80 22. The method of claim 18, wherein the contact between the collagenous tissue and the crosslinking reagent is effected by spraying.

23. The method of claim 1, further comprising the step of:  
periodically re-contacting the portion of the collagenous tissue with an  
85 effective amount of a crosslinking reagent.

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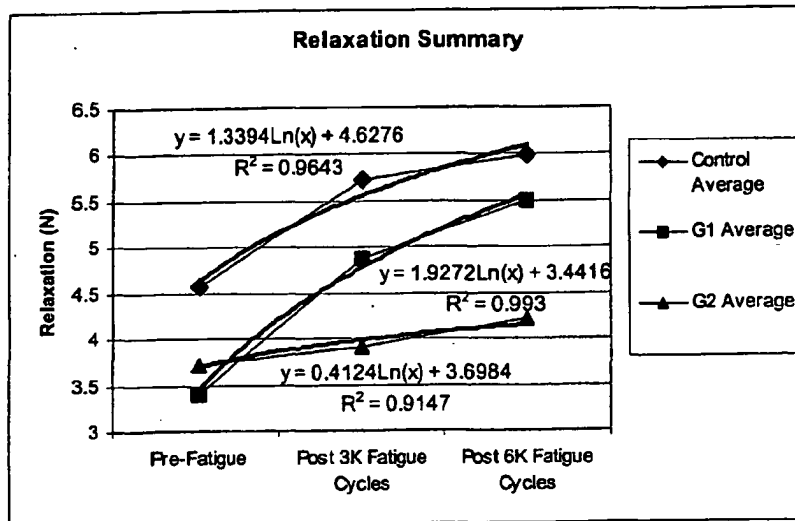


FIGURE 1

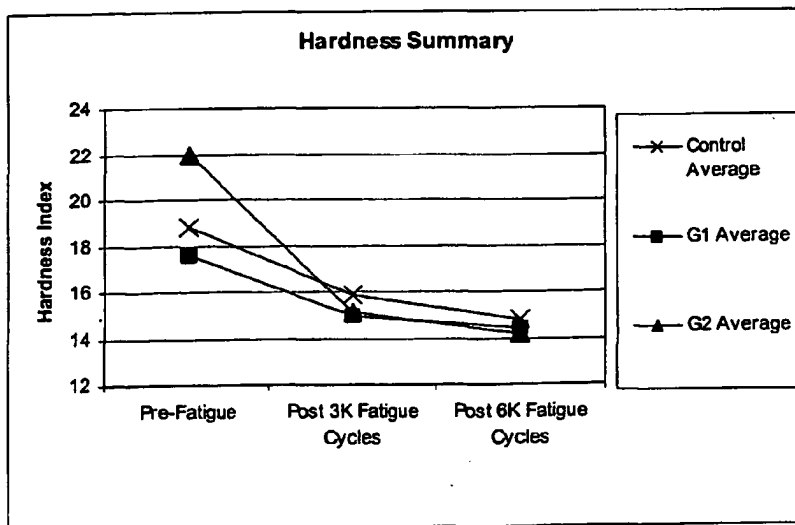


FIGURE 2



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/27677

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01N 43/04; A61K 31/70

US CL : 514/27

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
EAST, CAPLUS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,270,446 A (KYOGOKU et al) 14 December 1993 (14.12.1993), column 2, lines 16-40, claim 1, see entire document.	1, 2, 17
Y	WO 98/19718 A1 (CHALLENGE BIOPRODUCTS CO. LTD.) 14 May 1998 (14.05.1998), see entire document, page 3, lines 5-12; page 4, lines 25-30; page 6, line 5-33; claims 1-3 and 5-8.	1, 2, 17

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

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Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703)305-3230

Authorized officer

Valerie Bell-Harris for  
Ganapathy Krishnan

Telephone No. 703-308-1235